## REMARKS

Claims 65-66, 70-75, and 80-83 are pending in the application.

Claims 70-71, 75, and 80-83 were rejected under 35 U.S.C. § 112, first paragraph. Claims 65-66, 70, 72-74, and 83 were rejected under 35 U.S.C. § 103(a). Each of these issues is addressed as follows.

***Rejections under 35 U.S.C. § 112, first paragraph***

***New matter***

Claims 70 and 83 were rejected under 35 U.S.C. § 112, first paragraph as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventors had possession of the claimed invention. In particular, the Office states that the phrase "80% sequence identity with SEQ ID NO:4 within the CDR regions as identified in claims 73 and 80 represents a departure from the specification and the claims as originally filed....The homolog[s] were not described in terms of CDRs." For the following reasons, Applicants request reconsideration.

*Claim 70* reads:

> The pharmaceutical composition according to claim 65, wherein the variable region of said fragment comprises a sequence having at least 80% sequence identity with SEQ ID NO: 4 within the CDR regions as identified in Figure 13.

*Claim 83* reads:

> The antibody fragment of claim 72, wherein the variable regions of said fragment comprise a sequence having at least 80% sequence identity with SEQ ID NO: 4 within the CDR regions as identified in Figure 13.

As an initial matter, Applicants point out that one skilled in the art would appreciate that a "homolog" in the context of the present invention includes a

complementary determining region ("CDR"). Applicants' specification, for example, at page 28 (lines 26-27) teaches the CDR regions identified in Figure 13. Applicants further teach, for example, at page 29 (lines 1-2) that "antigen specificity ... is determined by the complementary determining regions." As is recognized by the skilled worker, a CDR is an antibody fragment. A CDR is also one example of a ligand, in the context of a homolog, as described at page 9 (line 26 et seq.) of the specification. Indeed, as noted in Applicants' previous response, the invention also envisions the use of ligands that include amino acid sequences. Furthermore, the specification at page 10 (lines 21-23), for example, states that "the present invention relates to ligands ... being derived from a monoclonal antibody." And the specification, at page 10 (lines 23-25) states: "the present invention also provides an antigen-binding Fab fragment, or a homolog or derivative of such fragment (emphasis added)." And CDRs are plainly antigen-binding Fab fragments. In view of Applicants' teaching, there is little question that claims 70 and 83 were disclosed in the specification where it is stated that "the ligands in accordance with the invention may include at least 80% sequence identity with the relevant ligand." In this case, the relevant ligands are the respective CDR regions identified in Figure 13. Accordingly, in view of these remarks, this basis of the section 112 rejection may be withdrawn.

***Enablement – Hybridoma Deposit***

Applicants include a Declaration by co-inventor, Dr. Hans Deckmyn, stating that the deposit, LMBP5108CB, shall be maintained for a term of at least thirty (30) years or five (5) years after the most recent request for the furnishing of a sample of the deposit was received by the Belgian Coordinated Collections of Microorganisms (BCCM™) or for the enforceable life of the patent for which the deposit was made, as well as indicating that any restrictions on the availability to the public of cell line LMBP5108CB will be irrevocably removed upon the granting of a patent on this application, with the exception of those restrictions listed in 37 C.F.R. § 1.808(b). This rejection should therefore be

withdrawn.

***Enablement – Scope***

Claims 70 and 83 were rejected on scope of enablement grounds. Applicants respectfully request reconsideration on this issue.

Claims 70 and 83 are recited above.

The Office has rejected these claims on the basis that, absent the ability to predict which antibodies would function as claimed; it would require a level of experimentation that is excessive and undue. Applicants respectfully disagree.

To support this ground of the rejection, the Office relies on Rudikoff, Panka, and Amit for the proposition that the antigen-binding characteristics of an antibody are affected by even minor amino acid sequence alterations. While Applicants do not generally take issue with these characterizations of the references, they do disagree with the assertion that the references provide a basis to question the enablement of Applicants' specification as evidence of undue experimentation.

First, the Federal Circuit has noted that, specifically in the antibody art, enablement is not negated by the necessity for some experimentation such as routine screening. *In re Wands* 858 F.2d 731, 8 U.S.P.Q.2d 1400 (Fed. Cir. 1988). The present invention, like *Wands*, involves routine screening of antibodies having the desired characteristics. As stated *Wands*, "a considerable amount of experimentation is permissible, if it is merely routine." Screening antibodies having the required level of percent identity would not require undue experimentation in the context of *Wands*.

Indeed, Rudikoff, Panka, and Amit each screened antigen-binding antibody variants. All of the references demonstrate that characterizing antigen-binding in view of amino acid sequence was routine at the time the application was filed. Also routine at that time was determining the level of identity to a reference sequence such as Applicants' disclosed CDRs. Although some variants may have weak antigen-binding characteristics this is not detrimental to enablement. Rudikoff makes clear, at page 1982

(col. 1), that "[A]ll such substitutions [single amino acid substitutions] need not and probably do not affect antigen binding." Panka simply shows that differences in the variable region framework resulted in decreased or increased affinity of variant antibodies. Similarly, Amit, using crystallography, characterized antigen-binding characteristics at the amino acid sequence level. All of these references amply demonstrate that routine methods for determining whether an antibody possesses the required features were known in the art at the time the application was filed. None of these references support the proposition that it would require undue experimentation to identify antibodies falling within the present claim scope.

Turning to the Office's additional concern that the specification provides no guidance how to produce monovalent antibody fragments that includes a sequence having at least 80% sequence identity with SEQ ID NO:4 within the CDR regions, Applicants point out that armed with Applicants' CDR sequences one skilled in the art could readily produce the claimed compositions. The Federal Circuit has long held that the proper test of enablement is "whether one reasonably skilled in the art could make or use the invention from the disclosures in the patent coupled with the information known in the art without undue experimentation (emphasis added)." *Hybritech, Inc. v. Monoclonal Antibodies, Inc.* 802 F. 2d. 1318 (Fed, Cir. 1985). Indeed, using standard computer programs and cloning methods, one skilled in the art could generate a number of amino acid substitutions within the CDR, and test for antigen-binding specificity and biological activity as outlined in Applicants' specification without undue experimentation. Again all that is involved is routine screening. This test for enablement has also been met in the present case.

For all of the above reasons, Applicants request reconsideration and withdrawal of this basis for the enablement rejection.

***Rejection under 35 U.S.C. § 103(a)***

Claims 65-66, 70, 72-74, and 83 were rejected under 35 U.S.C. § 103(a) as being

unpatentable over Ward et al. (1995) in view of Owens et al. (1994) and U.S. Patent No. 4,731,245 (the "'245 patent"). For the following reasons, Applicants respectfully traverse this rejection.

To establish a *prima facie* case of obviousness under § 103, the Office must demonstrate that the differences between the claimed invention and the prior art are such that the subject matter as a whole would have been obvious, at the time the invention was made, to a person having ordinary skill in the art. 35 U.S.C. § 103(a) (Supp. III 1997); *In re Dembiczak*, 175 F.3d 994, 998, 50 U.S.P.Q.2d 1614, 1616 (Fed. Cir. 1999), *abrogated on other grounds by In re Gartside*, 203 F.3d 1305, 53 U.S.P.Q.2d 1769 (Fed. Cir. 2000). Whether or not a claimed invention would have been obvious is a "legal conclusion based on underlying factual inquiries including: (1) the scope and content of the prior art; (2) the level of ordinary skill in the art; (3) the differences between the claimed invention and the prior art; and (4) objective evidence of nonobviousness." *Id.*

Where "claimed subject matter has been rejected as obvious in view of a combination of references, a proper analysis under § 103 requires, *inter alia*, consideration of two factors: (1) whether the prior art would have suggested to those of ordinary skill in the art that they should . . . carry out the claimed process; and (2) whether the prior art would have revealed that in so . . . carrying out, those of ordinary skill would have a reasonable expectation of success." *In re Vaeck*, 947 F.2d 488, 493, 20 U.S.P.Q.2d 1438, 1442 (Fed. Cir. 1991). Furthermore, it is "impermissible within the framework of section 103 to pick and choose from any one reference only so much of it as will support a given position, to the exclusion of other parts necessary to the full appreciation of what such reference fairly suggests to one of ordinary skill in the art." *In re Wesslau*, 353 F.2d 238, 241, 147 U.S.P.Q. 391, 393 (C.C.P.A. 1965) (emphasis added).

"Both the suggestion and the expectation of success must be founded in the prior art, not in the applicant's disclosure." *In re Dow Chem. Co.*, 837 F.2d 469, 473, 5

U.S.P.Q.2d 1529, 1531 (Fed. Cir. 1988). As the Federal Circuit has observed (emphasis added):

> A critical step in analyzing the patentability of claims pursuant to section 103(a) is *casting the mind back to the time of invention*, to consider the thinking of one of ordinary skill in the art, guided only by the prior art references and the then-accepted wisdom in the field. . . . *Most if not all inventions arise from a combination of old elements*. . . . Thus, every element of a claimed invention may often be found in the prior art. . . . However, identification in the prior art of each individual part claimed is insufficient to defeat patentability of the whole claimed invention. . . . Rather, to establish obviousness based on a combination of the elements disclosed in the prior art, there must be some motivation, suggestion or teaching of the desirability of making the specific combination that was made by the applicant.

*In re Kotzab*, 217 F.3d 1365, 1369-70, 55 U.S.P.Q.2d 1313, 1316 (Fed. Cir. 2000) (citations omitted) (emphasis added).

Moreover, the evidence of a suggestion, teaching, or motivation to combine "must be clear and particular (emphasis added)." *Dembiczak*, 175 F.3d at 999, 50 U.S.P.Q.2d at 1617. "Defining the problem in terms of its solution reveals improper hindsight in the selection of the prior art relevant to obviousness." *Monarch Knitting Mach. Corp. v. Sulzer Morat GMBH*, 139 F.3d 877, 881, 45 U.S.P.Q.2d 1977, 1981 (Fed. Cir. 1998). Thus, even if the Examiner identifies every element of a claimed invention in the prior art, this alone is insufficient to negate patentability. Otherwise, "rejecting patents solely by finding prior art corollaries for the claimed elements would permit an examiner to use the claimed invention as a blueprint for piecing together elements in the prior art to defeat the patentability of the claimed invention." *In re Rouffet*, 149 F.3d 1350, 1357, 47 U.S.P.Q.2d 1453, 1457 (Fed. Cir. 1998). To avoid hindsight based on the invention to defeat patentability of the invention, the Federal Circuit requires an Examiner to show a motivation to combine the references that create the case of obviousness. *Id.* That is, "the examiner must show reasons that the skilled artisan, confronted with the same problems as the inventor and *with no knowledge of the claimed invention*, would select

the elements from the cited prior art references for combination in the manner claimed." *Id.* (emphasis added).

In maintaining the obviousness rejection the Office states:

> It would have been obvious to one of ordinary skill in the art at the time the invention was made to produce the monoclonal antibody taught by Ward et al as Fab as taught by the Owens *et al* and place the resultant Fab fragment which binds to platelet glycoprotein GPIbα polypeptide taught by the Ward et al reference in a composition taught by the ' patent.
>
> One of ordinary skill in the art at the time the invention was made would have been motivated to do so because eight antibodies mapped to the N-terminal fragments of gpIba, and these were tested for their ability to block binding of $^{125}$ I-labelled von Willebrand factor to washed platelets in the presence of ristocetin or botrocetin and because it would further lead to insights into mechanisms of vWF function both in vitro and in vivo. Given that the antibody fragments are the reagents of choice for some clinical applications one ordinary skill in the art at the time the invention was made would be motivated to include such fragments in a composition because the composition can be formulated in dosage unit form. The amount of the active ingredient contained in each dosage unit may be adjusted so as to enable the administration of the antibody at a daily dose as taught by '245 patent.

This rejection should be withdrawn because the asserted motivation for combining these references is unsupported by the references themselves. No *prima facie* case for obviousness exists in this case.

It is inappropriate for the Office to view Owens, Ward and the '245 patent in hindsight and with the success of Applicants' invention in mind. Instead, what is required is that the Office must "cast its mind back to the time of the invention," as required by *In re Dow* and be guided "*only* by the prior art references and the then-accepted wisdom in the field." *In re Dow*, 837 F.2d at 473 (emphasis added).

If this standard is applied, it is clear that a skilled worker reading Ward would not be motivated to look to Owens or the '254 patent or both. First, Ward never indicates that one of the 17 monoclonal antibodies that bind GB1bα would be beneficial for

therapeutic administration. Ward makes no mention of antibody fragments, and further never teaches or suggests that fragments of the anti-GB1bα antibodies be produced. Ward is also uncertain as to the significance of their own findings, concluding "**[f]urther studies** ... ***may provide valuable insights into the mechanisms of vWF function*** *in vitro* and *in vivo*." What are the valuable insights into the mechanisms of vWF function? At best, Ward advocates additional research. Wards provides no "clear and particular" direction for using any of the eight disclosed antibodies as therapeutics.

With respect to Ward, the Office also asserts that "Ward has done nothing different [than] the Applicant's specification with respect to in vivo data." Applicants disagree. Unlike Ward, which provides no opinion on *in vivo* function of the anti-GB1b antibodies, Applicants teach, for example, *in vivo* baboon studies (Examples 7-9).

Owens and the '245 patent never mention antibodies that bind human glycoprotein GP1b much less suggest that the claimed antibodies would be beneficial therapeutics. Both references are unavailing because each merely provides methods known at the time the application was filed. None, alone or in combination with Ward, provides a single insight into the *in vivo* therapeutic activity of the claimed compositions.

The Ward, Owens, and '245 patent prior art combination is not the type of "clear and particular" motivation required by the Federal Circuit. *In re Dembiczak*, 175 F.3d at 999. Without motivation for the combination of references, no *prima facie* case of obviousness can exist, and the § 103 rejection on this basis alone must be withdrawn.

The Office attributed little weight to Applicants' reliance on the Cadroy and Bergmeier references, stating:

> Both references used either an intact antibody or a divalent antibody, but not monovalent antibody. Again the resultant antibody fragment of Fab or scFv would not be expected to cause thrombocytopenia.

Bergmeier, makes clear, at page 892 (2nd paragraph) that "attempts to block certain epitopes on GP1b with modified antibodies may generally result in thrombocytopenia....[and] in vivo blockage of certain epitopes on GP1b may, therefore,

not be a promising antithrombotic strategy." Bergmeier teaches that antibody binding to GP1b results in thrombocytopenia, regardless of whether the antibody is a $F(ab)_2$, Fab, or scFv. Indeed, Bergmeier concedes that thrombocytopenia results from the binding between the antibody and its GP1b epitope.

Bergmeier discourages one skilled in the art away from Applicants' monovalent antibody fragment antithrombotic strategy. Bergmeier's teaching would lead a person of ordinary skill, upon reading the reference, in a direction divergent from the path that was taken by Applicants. The skilled worker would not be led to construct additional antibody fragments such as Fabs or scFvs when Bergmeier teaches that antibody binding to GP1b results in thrombocytopenia. For this reason as well, the obviousness rejection in this case must be withdrawn. See, for example, *In re Haruna*, 249 F.3d 1327, 1335, 58 U.S.P.Q.2d 1517, 1522 (Fed. Cir. 2001) ("A prima facie case of obviousness can be rebutted if the applicant ... can show 'that the art in any material respect taught away' from the claimed invention .... *A reference may be said to teach away when a person of ordinary skill, upon reading the reference, ... would be led in a direction divergent from the path that was taken by the applicant.*")(emphasis added) (citations omitted).

Indeed, as further evidence that use of antibodies against GP1b as therapeutics was discouraged by workers in the field, applicants direct the examiner's attention to Phillips et al. (Therapeutic approaches in arterial thrombosis, *Journal of Thrombosis and Haemostasis* 3:1577-1589, 2005; copy enclosed) at page 1583, left column, where it is stated that "[m]odulation of the VWF/GPIbα axis has been the subject of many investigations with promising animal experimental results, but severe thrombocytopenia has been associated with the use of antibodies against GPIbα, thus reducing the general interest of the scientific community for several years." Given this statement too, it is unreasonable to assume that one skilled in the art, at the time the application was filed, would have been motivated to combine the teachings of Ward, Owens, and the '245 patent in the manner suggested by the Office.

Furthermore, the Office, provides no authority, as required by the Federal Circuit,

for the conclusion that Fab or scFv fragments would "not be expected to cause thrombocytopenia" in the context of an antibody that binds to GP1b. See, for example, *In re Sang Su Lee,* 277 F.3d 1338, 1343, 61 U.S.P.Q.2d 1430, 1433 (Fed. Cir. 2002), *quoting McGinley v. Franklin Sports, Inc.*, 262 F.3d 1339, 1351-1352, 60 U.S.P.Q.2d 1001, 1008 (Fed. Cir. 2001). ("[T]he factual question of motivation [to combine references] is material to patentability, and ...[cannot] be resolved on subjective belief and unknown authority.").

Finally, the Office's reliance on an inherent characteristic of the resultant antibodies (i.e, "without incurring thrombocytopenia") is also contrary to Federal Circuit case law. In *In re Rijckaert*, 9 F.3d 1531, 28 U.S.P.Q.2D 1955 (Fed. Cir. 1993), the court faced with an equivalent question unambiguously held that:

> "The mere fact that a certain thing may result from a given set of circumstances is not sufficient [to establish inherency.]" *In re Oelrich* , 666 F.2d 578, 581-82, 212 USPQ 323, 326 (CCPA 1981) (citations omitted) (emphasis added). "That which may be inherent is not necessarily known. Obviousness cannot be predicated on what is unknown." *In re Spormann*, 363 F.2d 444, 448, 150 USPQ 449, 452 (CCPA 1966). Such a retrospective view of inherency is not a substitute for some teaching or suggestion supporting an obviousness rejection. *In re Newell*, 891 F.2d 899, 901, 13 USPQ2d 1248, 1250 (Fed. Cir. 1989)

The holding goes directly to the issue of the appropriateness of the Office's obviousness rejection in view of its finding that the "resultant antibody fragment ... would not be expected to cause thrombocytopenia." The Office provides no support for its inherency theory. Applicants' claim limitation "without incurring thrombocytopenia" is neither "inherent" in Ward and Owens, nor the '245 patent. In short, the Office's retrospective view of inherency is not a substitute for some teaching or suggestion which supports the selection and use of the claimed composition. For this reason too, Applicants respectfully request reconsideration of the obviousness rejection

## CONCLUSION

Applicants respectfully request reconsideration and withdrawal of all rejections in this case.

Enclosed are a Petition to extend the period for replying to the Office Action for three (3) months, to and including November 28, 2005, and a check in payment of the required extension fee. Also enclosed is a Notice of Appeal.

If there are any additional charges or any credits, please apply them to Deposit Account No. 03-2095.

Respectfully submitted,

Date: 28 November 2005

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REVIEW ARTICLE

# Therapeutic approaches in arterial thrombosis

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**Summary.** The current standard of care for the treatment of arterial thrombosis includes anticoagulants and three classes of antiplatelet agents – aspirin, thienopyridines and glycoprotein IIb-IIIa antagonists. Although these drugs have had a significant impact on morbidity and mortality in several patient populations, up to 15% of the high risk patients with acute coronary syndrome continue to suffer from ischemic events. This problem may occur, in part, because the platelets in many patients are non-responsive to aspirin and clopidogrel. Murine models now indicate that platelets are not only responsible for arterial occlusion, they are also involved in the progression of atherosclerotic disease. New opportunities have emerged identifying potential targets and strategies for drug discovery suited to address these deficiencies by more effectively modulating platelet adhesion, thrombus growth, thrombus stability and the pro-inflammatory activity of platelets. In addition, a growing need has emerged for the development of bedside devices capable of bringing personalized medicine to patients being treated with antithrombotic drugs in order to measure the pharmacodynamic activities of new therapies, to assess the activities achieved by combined antithrombotic therapy, and to identify patients that fail to respond.

**Keywords:** anticoagulant, antiplatelet, atherosclerosis, platelet, platelet monitoring, thrombosis.

## Introduction

Arterial thrombosis is the result of sequential events involving platelet adhesion, activation and subsequent aggregation that can lead to vascular occlusion, perhaps the primary pathological complication of advanced atherosclerotic lesions. Recent advances in the field of thrombosis suggest that the second pathological consequence of platelet adhesion and activation may be as consequential as the immediate ischemia induced by arterial thrombosis as platelets are a primary source of several inflammatory proteins known to be involved in the progression of atherosclerotic disease including RANTES, sCD40L, PDGF and transforming growth factor-β (TGF-β). These considerations suggest that therapeutic targeting of platelets has two objectives: first, prevention of vessel occlusion; second, inhibition of the platelet contribution to lesion progression.

The pharmaceutical industry has made important inroads into the development of drugs for the treatment of the thrombotic complications of atherosclerosis. In one example, the occlusive, ischemic consequences of acute myocardial infarction (MI) have been addressed, by thrombolytics to lyse the thrombus, and more recently by interventional strategies to mechanically remove or dislodge the thrombus and to maintain artery patency with stents coated with agents such as rapamycin or paclitaxel to reduce the incidence of restenosis. In yet another example, antagonists against platelet receptors such as glycoprotein (GP) IIb-IIIa and $P2Y_{12}$ have been developed, joining aspirin, a Cox-1 inhibitor as the primary antithrombotic drugs. However, despite these advances in antithrombotic therapies and the widespread use of statins to reduce cholesterol and CRP levels, the incidence of atherosclerosis continues to rise, as do the ischemic consequences of atherosclerosis including MI and stroke. An added complication is type 2 diabetes which is an independent risk factor for cardiovascular disease (CVD). A recent analysis of the Framingham Heart Study showed that even though management of risk factors such as blood pressure and cholesterol has improved significantly in the total patient population, the presence of diabetes significantly reduced the overall benefits [1]. While it may be possible to address these pathologies by more aggressive health management, and by more optimal application of existing therapies, clearly, truly effective treatment of the thrombotic consequences of atherosclerosis requires not only the development of drugs to be used as primary care on a chronic basis to prevent thrombosis and its ischemic complications but also to block the contribution of platelet-induced inflammation in the progression of atherosclerotic disease. Reviewed below is the mechanism of action, clinical successes and limitations of the four drug classes currently used to prevent arterial thrombosis; aspirin, $P2Y_{12}$ inhibitors, GP IIb-IIIa antagonists and anticoagulants. Therapeutic opportunities afforded by our current understanding of the mechanisms of arterial thrombosis and the inflammatory activity of platelets are discussed. Finally, recognizing that individuals vary in response to various drugs and that combination antithrombotic therapies has become commonplace, we will highlight the need for improved pharmacodynamic assessment of platelet function.

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## Current strategies

Current therapeutic strategies for the treatment of arterial thrombosis are based on the well-known receptor systems summarized in Fig. 1. In this simplified diagram, collagen and/or thrombin are designated as the primary platelet agonists. While either agonist is capable of activating platelets, including the activation of the receptor function of GP IIb-IIIa for the binding of fibrinogen and von Willebrand factor (VWF) to initiate platelet aggregation, stable aggregation of platelets is augmented by two autocrine factors generated upon platelet stimulation: ADP, released from platelet dense bodies, and $TXA_2$, generated by the sequential actions of Cox-1 and thromboxane synthase on the arachidonic acid released from membrane phospholipids. Additional aggregation-dependent secondary mediators include sCD40L and Gas6 plus aggregation-induced tyrosine phosphorylation of GP IIIa and activation of secondary aggregation receptors such as SLAM, CD84, Eph kinase and the Gas-6 receptors. Even though the signaling reactions induced by the receptor systems for the platelet stimuli summarized in Fig. 1 are diverse, including those coupled by $G_q$ (PAR-1 and TP), $G_i$ ($P2Y_{12}$), Syk (GP VI), Shc and talin (GP IIb-IIIa), drugs that target these receptor systems have been designed either to specifically inhibit the receptors themselves [e.g. GP IIb-IIIa antagonists (eptifibatide, abciximab, tirofiban) and $P2Y_{12}$ inhibitors (clopidogrel, ticlopidine)], to block the generation of the agonists (e.g. the Cox-1 inhibitor aspirin and Factor Xa (FXa) inhibitors [low molecular weight (LMW) heparins]), or to antagonize the agonist itself (e.g. thrombin inhibitors (standard heparin, direct thrombin inhibitors)).

### *Aspirin*

The clinical successes achieved by the current therapies to treat arterial thrombosis have been remarkable. Aspirin was the first and continues to be the most widely used of these drugs. The trend toward the widespread use of this drug to block arterial thrombosis was first indicated by the findings of the ISIS-2 trial which demonstrated that aspirin reduced mortality from acute MI to a rate that is similar to that of the thrombolytic agent, streptokinase [2]. The data from multiple trials summarized by the Antiplatelets Trialist's Collaboration found a 25% relative risk reduction by aspirin of vascular death, MI or stroke, vs. placebo [3] which led to the widespread adoption of aspirin as standard therapy for primary and secondary prevention of arterial ischemia. This collaboration also reviewed the clinical trials using aspirin to show that low-dose aspirin (75–150 mg daily) is effective for long-term use [4]. While the half-life of aspirin in humans is relatively short (~20 min), its effect persists for the lifetime of an affected platelet in circulation as the drug acetylates Cox-1 at serine-529, located at the active site of the enzyme. Attempts have been made to develop additional drugs that target the thromboxane pathway in platelets including a variety of thromboxane receptor (TP) antagonists, thromboxane $A_2$ synthase inhibitors, or compounds that combined both functions [5]. Although some of these agents had potent antithrombotic effects in experimental models and preclinical studies, and offered the advantage of inhibiting the TP stimulating activity of prostanoid metabolites in addition to $TXA_2$ (e.g. isoprostanes, $PGH_2$), they are not currently used to block arterial ischemia as most were not evaluated in clinically relevant phase III trials [6].

### *Thienopyridines*

The second most widely used of the antiplatelet drugs for chronic use are thienopyridines targeting $P2Y_{12}$. This class of drugs, which includes clopidogrel, and its predecessor ticlopidine, act via irreversible inhibition of the platelet $P2Y_{12}$ receptor. Both are prodrugs, requiring hepatic metabolism by

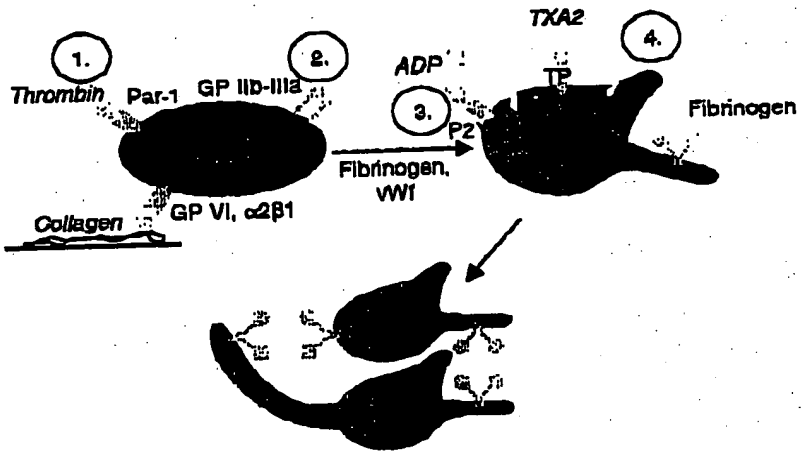


Fig. 1. Current therapeutic strategies for the treatment of arterial thrombosis. The reaction diagram is designed to illustrate the pathways regulated by current therapeutic strategies on unstimulated, discoid platelets (top left), stimulated platelets (top right), and aggregated platelets (bottom). The four antithrombotic drug classes include: (1) Thrombin inhibitors; (2) GP IIb-IIIa antagonists; (3) $P2Y_{12}$ antagonists; and (4) inhibition of $TXA_2$ production.

cytochrome P450 isoform 3A4 in order to generate the active metabolite, a transient intermediate which covalently modifies and inactivates the receptor. Ticlopidine has been shown to be efficacious in conditions such as claudication, unstable angina, and cerebrovascular disease [7]. However, the incidence of neutropenia associated with ticlopidine led to the development of a second-generation thienopyridine, clopidogrel, with increased potency and fewer side-effects. In the CAPRIE trial [8], clopidogrel was shown to be more efficacious than aspirin, particularly in high-risk patients (diabetics and those with a history of prior revascularization). Subsequently, the CURE study [9] demonstrated that patients with unstable angina or non-ST segment elevation MI received a 20% relative risk reduction if they were randomized to clopidogrel plus aspirin vs. placebo plus aspirin, and the PCI-CURE substudy [10] showed that this benefit extended to patients undergoing percutaneous intervention (PCI). The slow onset of action of thienopyridines, due to their metabolism requirement, has necessitated the administration of a large loading dose (300 mg) prior to acute procedures, such as PCI, as demonstrated in the CREDO trial, where the maximum benefit of clopidogrel administered with aspirin required a loading dose given at least 6 h prior to the procedure. This study also demonstrated a significant 27% reduction in death, MI and stroke from 1-year administration of clopidogrel plus aspirin following PCI, compared to 1-month dosing [11].

### GP IIb-IIIa antagonists

The GP IIb-IIIa antagonists are designed to bind to the integrin on unstimulated platelets and on platelets after stimulation. GP IIb-IIIa is an attractive antiplatelet target as it is (i) on the 'final common pathway' mediating platelet aggregation irrespective of the agonist used to induce platelet activation, (ii) platelet-specific, and (iii) responsible for a variety of aggregation-dependent platelet functions including those in coagulation, inflammation, fibrinolysis and vascular cell proliferation. Three GP IIb-IIIa antagonists have been developed: integrilin, a cyclic heptapeptide modeled after the active site of the disintegrin found in the southeast pigmy rattle snake; abciximab, a Fab fragment of a mouse/human chimeric antibody against GP IIb-IIIa; and tirofiban, a synthetic inhibitor of GP IIb-IIIa. All were designed to be infusible i.v. drugs and are therefore only administered to patients in acute settings who have a high risk of experiencing an ischemic event such as those undergoing PCI (with or without stent placement) or those with symptoms resulting from acute coronary syndrome (ACS) [12]. Use of these drugs has shown a remarkable reduction in death and MI for these indications [13–15].

### Anticoagulants

ACS patients, whether undergoing an invasive revascularization procedure or not, are treated with aspirin and antithrombin therapy in the form of unfractionated or LMW heparins. Although unfractionated heparin is effective in reducing clinical events, a narrow therapeutic index makes it a less than optimal antithrombin for this class of patients. Like their parent anticoagulant, i.e. standard heparin, LMW heparins are indirect antithrombins and utilize antithrombin III to mediate inhibition of thrombin and FXa. As LMW heparins have more predictable pharmacokinetics than standard heparin, they are used in a fixed dose manner. Early trials of the LMW heparin enoxaparin in unstable angina and non-Q wave MI patients demonstrated improved efficacy over standard heparin and the drug has emerged as the most commonly used LMW heparin [16,17]. Fondaparinux, a synthetic pentasaccharide, also utilizes the antithrombin III binding region of heparin and has been found to be an appropriate anticoagulant for prevention of deep vein thrombosis in orthopedic surgery [18]. Unlike enoxaparin, which inhibits both thrombin and FXa, fondaparinux acts only as an indirect FXa inhibitor. Venous thromboembolism prevention trials showed that fondaparinux has a superior efficacy profile to its comparator enoxaparin. Ongoing trials of fondaparinux in ACS patients will show if the concept of attaining superior efficacy by inhibition of FXa alone (vs. the combination of FXa and thrombin) can be achieved in arterial settings.

### Combination antithrombotic therapy

Arterial thrombosis developed at sites of spontaneously or mechanically disrupted atherosclerotic plaque is triggered by a multitude of highly thrombogenic materials (i.e. fibrillar collagen and tissue factor). It is the result of complex interrelations between coagulation and platelets orchestrated by local rheological conditions. An emerging strategy in the treatment of arterial thrombosis came with the realization that combinations of antithrombotics provide greater therapeutic benefit than are provided by drugs used singly. Accordingly, the combination aspirin-plus-clopidogrel is rapidly becoming the new standard of care for the management of patients with non-ST segment elevation ACS and in patients undergoing a PCI. In support of this trend, the CURE study demonstrated that aspirin-plus-clopidogrel caused a 20% relative risk reduction of vascular death, MI, and stroke compared with aspirin-plus-placebo [9]. The dual antiplatelet therapy (aspirin-plus-clopidogrel) was also more effective and safer than a combination aspirin-plus-warfarin in coronary artery stenting [19,20]. The remarkable efficacy of the dual anti-platelet therapy has prompted the initiation of several clinical trials in indications as diverse as atrial fibrillation, peripheral arterial disease, peripheral arterial bypass surgery, secondary and high-risk primary prevention, acute ST-segment elevation MI and heart failure [21]. Finally, although anticoagulants were routinely used in the development of antiplatelet agents, analysis of these data shows that these combinations often provided a clinical benefit that was greater than anticipated. We and others have used thrombosis models to evaluate synergisms between various pathways. Because $TXA_2$ and ADP activate different pathways, it was anticipated that combinations of inhibitors of the two pathways would confer a

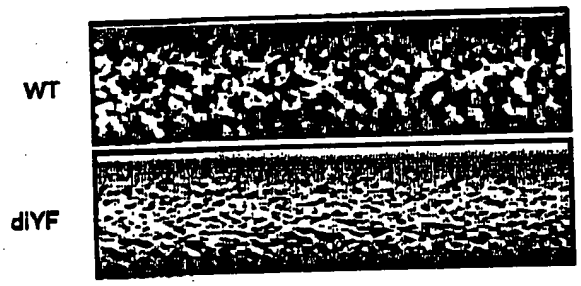


Fig. 2. Thrombosis defect in platelets from the diYF mouse. Unanticoagulated blood from a control mouse (WT) or from a mouse harboring the diYF mutation in GP IIIa (Y747F, Y759F) was perfused for 2.5 min through a chamber coated with type III collagen at 871 $s^{-1}$ and stained. The view is en face.

greater protection against thrombotic events. However, rather than an additive effect, the two drugs used together were synergistic [22,23]. A potent synergism between clopidogrel and anticoagulants [using either a direct thrombin inhibitor (Bivalirudin), a FXa inhibitor or a synthetic LMW heparin] has also been observed (Fig. 2) [23,24]. While these results may have a simple explanation, resulting from the inhibition of fibrin formation by the anticoagulant, where fibrin contributes to thrombus stability under arterial shear rate [25], the emerging data indicate that the anti-thrombotic synergisms may originate from the complementation of the signaling pathways in platelets. Activation of the receptor function of GP IIb-IIIa is optimal when engagement of $G_{12/13}$ or $G_q$ signaling pathways is combined with $G_i$ stimulation [26,27]. This combination occurs when either $TXA_2$ (TP) receptor ($G_{12/13}$, $G_q$), protease-activated receptor-1 (PAR-1) ($G_{12/13}$, $G_q$), and also potentially $P2Y_1$ ($G_q$) [28] is allowed to synergize with that of $P2Y_{12}$ ($G_i$). Note that this model might also explain the synergism between aspirin and clopidogrel. A different explanation may stem from the inhibition of the different PI-3 kinase enzymes present in platelets. For example, $P2Y_{12}$ engagement by ADP stimulates PI-3 kinase-γ whereas the engagement of either FcγRIIA/GPVI, PAR-1, TP, or GP IIb-IIIa lead to PI-3 kinase α or β-activation. This could indicate that any combination therapy that would block PI-3 kinase α, -β or -γ would confer a strong antithrombotic efficacy.

## Limitations of current antithrombotics

Even with the remarkable successes that have been achieved with currently available antithrombotics in the prevention of arterial thrombosis, limitations of this class of drugs do exist. It is valuable, therefore, to consider additional strategies currently available to design new drugs that address these limitations. That there is room for improvement is readily apparent from analysis of current trials. For example in SYNERGY, a trial with more than 10 000 high risk non-ST segment elevation ACS patients treated with heparin or LMW heparin, aspirin, and, as determined by the physician, clopidogrel and/or GP IIb-IIIa antagonists, approximately 15% of all patients still experienced death or non-fatal MI within 30 days of treatment [29]. Except for aspirin, perhaps the biggest limitation of these drugs is that the dose used is less than optimal for the treatment of thrombosis. The drugs were typically titrated in preclinical development studies to arrive at a dose that had a significant inhibition of thrombosis without undue bleeding. While higher doses of the drugs had better antithrombotic activity, this always created a bleeding risk. Although the dose selected required some refinement in subsequent clinical studies, this same strategy was employed.

### *Aspirin non-responsiveness*

While aspirin is used at a dose (e.g. 70–325 mg $day^{-1}$) that yields near 100% acetylation of Cox-1 in most individuals, it has been recognized for several years that individuals treated with aspirin still experience thrombotic events. The interpretation of this observation has been controversial. One could argue that aspirin is a comparatively 'weak' inhibitor in that it only blocks the production of $TXA_2$, an autocrine factor that supplements the activities of the primary platelet agonists. Indeed, aggregation reactions using platelets from individuals taking aspirin appear normal when high concentrations of primary agonists are used. However, it has become clear from recent pharmacodynamic and biochemical studies that platelet responses normally blocked by inhibition of Cox-1 (the target of aspirin in platelets) are still present in some individuals, an observation that has led to the concept of 'aspirin resistance' or 'aspirin non-responders' [30–32]. Studies of platelet aggregation in aspirin-treated CVD patients, both by traditional ADP and arachidonic acid induced aggregation studies and by platelet function analyzers such as PFA100, show that ≤ 10% of the individuals can be classified as aspirin non-responders [31]. Additional studies demonstrated a threefold higher risk of major cardiovascular adverse events in the same patient population [30]. As the incidence of aspirin non-responders had been shown to be higher in patients who undergo coronary artery bypass graft (CABG), a recent study evaluated the functional and biochemical responses to aspirin on subsequent days following a CABG procedure in a small number of patients [33]. The study demonstrated that platelets from these patients after CABG did not completely respond to aspirin *in vitro*, and that while Cox-1 levels in platelets remained constant 10 days following the procedure, there was a pronounced increase in the level of Cox-2, which is ~170-fold less sensitive to aspirin inhibition, especially at 5 days post-procedure. This may be reflective of the increased platelet turnover following cardiopulmonary bypass, and the increased level of Cox-2 could generate critical amounts of $TXA_2$, in spite of aspirin treatment, providing a possible explanation for aspirin non-responsiveness. Other mechanisms proposed as contributing factors to aspirin non-responsiveness include use of NSAIDs, which block acetylation by aspirin [34] and polymorphisms of platelet genes (Cox-1 or GP IIb-IIIa) [35], and non-compliance. However, the answer may be totally unexpected such as the activation of a deacetylase. Clearly, further

studies are needed to better define the underlying mechanisms of this phenomenon. Aspirin use is also contraindicated in a significant population of patients, i.e. those with gastrointestinal bleeding and those with aspirin-induced asthma.

### *Clopidogrel non-responsiveness*

The primary limitation of clopidogrel is that this drug demonstrates weak and somewhat variable inhibition of $P2Y_{12}$ [36]. Following a 600-mg loading dose of clopidogrel, the extent of inhibition of ADP-induced aggregation (5 μM ADP) varied from 33% to 78% in healthy individuals, at 6 h post-dosing [37]. This effect is further exaggerated in patients undergoing PCI/stent placement [38,39]. The antithrombotic effect of clopidogrel is likely to be dependent on a number of factors including but not limited to variations in P450s, polymorphisms of the $P2Y_{12}$ receptor and receptor signaling pathways. Measurements of platelet aggregation and markers of platelet activation (GP IIb-IIIa and P-selectin detection by specific antibodies) show that clopidogrel resistance is detected in 31% of the patients on day 5 and 15% of the patients on day 30 of the treatment regimen [40]. A prospective study of PCI patients with non-ST segment elevation MI showed that up to 25% of the patients were resistant to clopidogrel [41]. When the patients were stratified into quartiles based on resistance to ADP-induced platelet aggregation, the most resistant patients had a 40% adverse event rate during a 6-month follow-up period so they are obviously being denied adequate protection based on inhibition of $P2Y_{12}$. It has also been reported that the antiplatelet activity of clopidogrel is blocked in patients treated with a widely used cholesterol lowering medication (atorvastatin) which is undoubtedly linked to the metabolism requirement for efficacy [42]. A second limitation of these drugs stems from their irreversible mechanism of action, which inactivates the $P2Y_{12}$ receptor for the lifetime of the platelet. While this is not a particular problem with aspirin, which is also irreversible, this feature has led to limited use of clopidogrel before PCI in patients who are at increased risk of undergoing CABG procedures, as the risk for bleeding following clopidogrel treatment requires postponement of the procedure for 5–7 days, or transfusion of large numbers of platelets during the procedure.

### *Limitations of anticoagulants – i.v.*

Each anticoagulant has evolved unique issues. Replacement of unfractionated heparin has had mixed success. Although the current ACC/AHA guidelines (2002) prefer enoxaparin over unfractionated heparin, recent data do not support this preference. In the SYNERGY trial, enoxaparin was found not to be superior to unfractionated heparin [29]. In A to Z, for patients on a GP IIb-IIIa antagonist (tirofiban) and aspirin, enoxaparin was found to be non-inferior to standard heparin [43]. LMW heparins also have a narrow safety window. For example, in unstable angina patients treated with enoxaparin, a 25% dose increase in therapeutic level (1.25 mg $kg^{-1}$ vs. 1 mg $kg^{-1}$) produces an unacceptable number of bleeding events [44]. Direct thrombin inhibitors such as angiomax have also been studied as replacements for unfractionated heparin. In the REPLACE 2 trial of PCI, angiomax and provisional GP IIb-IIIa inhibitor compared favorably to heparin plus GP IIb-IIIa [45]. The primary end point of the trial combined efficacy and safety parameters and the angiomax arm of the trial was statistically not inferior to the heparin arm. However, the benefit related to reduction of bleeding with the use of angiomax is questionable. In REPLACE 2, the control group was likely to have been over anticoagulated as the observed ACT values in the heparin arm were higher than the recommended ACT range (200–300 s) for use of heparin in conjunction with GP IIb-IIIa. As angiomax is substantially more expensive than standard heparin, economic considerations also contribute to its limited use in PCI patients.

### *Limitations of anticoagulants – oral*

Warfarin is the only anticoagulant in chronic use. While the drug provides tremendous benefit to affected individuals, its anticoagulant response is influenced by a variety of factors such that >50% of patients are usually outside of the therapeutic range. Due to the large variability in the anticoagulant effect of warfarin and its narrow therapeutic index, a large unmet clinical need exists for an anticoagulant with predictable fixed-dose usage. The need for a warfarin substitute has led to numerous drug development projects that have focused on inhibitors of coagulation proteases that specifically inactivate the protease active site. Ximelagatran, an oral thrombin inhibitor, was the first to show that the strategy of direct coagulation protease inhibition does translate into effective anticoagulation and leads to antithrombotic activity in deep vein thrombosis and atrial fibrillation patients [46]. Two studies suggest that ximelagatran is at least as effective as warfarin in preventing stroke in high-risk patients with atrial fibrillation [47]. The studies also showed that there are incidences of increase in liver enzymes which would require surveillance for potential liver toxicity in future patients. Unfortunately, safety problems of ximelagatran related to serious liver toxicity has led the FDA to recommend against approval of this thrombin inhibitor. While several new experimental agents with the potential to be an effective and low variability anticoagulant have been evaluated in clinical trials, none of these are available for therapeutic use, so the search for a warfarin replacement remains a work in progress.

### *PAR-1*

While drugs that inhibit thrombin or prevent its formation are a mainstay in the armamentarium used for the treatment of arterial thrombosis, as of this writing, no efficacy trials have been performed to determine whether antagonists of PAR-1, the thrombin receptor on platelets, could provide a therapeutic benefit. Potent and selective PAR-1 antagonists capable of inhibition of thrombin-induced platelet aggregation have been

reported in the literature. Peptide mimetic antagonists such as RWJ-58259 are effective in models of thrombosis and vascular injury and could have potential as therapies for treating thrombosis and restenosis [48]. An oral PAR-1 antagonist, E-5555, is being developed as a drug candidate for ACS but definitive clinical efficacy trials have not been reported [49].

## Future directions for antithrombotic drug development

New antithrombotics are required not only to overcome the limitations of the current drugs to better manage arterial ischemia, but also to address the inflammatory activities of platelets which contribute to progression of atherosclerotic disease. The successes and limitations of current therapies coupled with the advances made in our understanding of platelet biology are instructive in the design of new drugs to more effectively regulate validated targets, in the identification of new targets that may safely provide increased benefit and in the development of the proper combination of antithrombotics for the various arterial ischemic indications.

### *Agonist receptors*

While platelets are activated by numerous agonists acting on multiple receptors, the only validated agonist receptor for drug discovery is $P2Y_{12}$. The requirements for improvements over clopidogrel are clear – more potent inhibition of $P2Y_{12}$; less variability of inhibition between different patients; no requirement for metabolism resulting in less delay in onset to action; and quicker recovery of platelet function following discontinuation of use. While these requirements can most likely be best achieved by an orally available reversible $P2Y_{12}$ antagonist, preclinical data indicate three promising candidates in development with different properties. One is cangrelor (AR-C69931MX), a nucleotide, intravenous compound that reversibly antagonizes $P2Y_{12}$ [50]. AZD-6140, an orally available direct-acting $P2Y_{12}$ antagonist [51], is presently being evaluated in phase II clinical trials. Prasugrel (CS-747), a thienopyridine prodrug similar to clopidogrel which is more rapidly converted to the active metabolite than is clopidogrel, has completed phase II trials and will be evaluated in phase III trials in ACS patients [52].

ADP also acts on $P2Y_1$, a $G_q$ coupled receptor. Studies using either selective antagonists of $P2Y_1$ or $P2Y_{12}$, as well as gene-targeting strategies [53–56] have demonstrated distinct roles for these two ADP receptors. $P2Y_1$ is responsible for initiation of aggregation to ADP [55], while $P2Y_{12}$ is critical for amplification of the aggregation response by released ADP, and for stabilization of platelet aggregates and the growing thrombus [57]. In addition to the different roles of these two receptors in initiation and stabilization of thrombus growth, one could argue that the selective tissue distribution of $P2Y_{12}$ (platelets, megakaryocytes and glial cells), vs. $P2Y_1$ (which is ubiquitously expressed) makes it the preferred drug target. Although there are selective $P2Y_1$ antagonists which have been used as *in vitro* tools [58,59], none of these have been clinically evaluated as yet, and may not have suitable pharmacokinetic properties to be viable drug candidates.

### *Secondary aggregation receptors*

While the initial interaction of platelets during thrombosis is dependent upon GP IIb-IIIa, it has become apparent that signaling reactions initiated by platelet–platelet contact are required for thrombus stability. Several mediators of aggregation-induced signals have been identified. One is GP IIb-IIIa itself which becomes tyrosine phosphorylated and also associates with numerous signaling and cytoskeletal proteins following platelet aggregation. The importance of the 'outside-in' signaling in the enhancement of platelet aggregation was demonstrated by the generation of knock-in mice where tyrosine residues Y747 and Y759 were mutated to phenylalanine [60]. The so-called DiYF mice displayed selective impairment of outside-in signaling resulting in the formation of unstable aggregates. In addition, as shown in Fig. 2, *ex vivo* perfusion chamber experiments on type III collagen have shown that the DiYF mouse has defective thrombosis. Another protein involved in secondary platelet aggregation is CD40L, a tumor necrosis factor family member mainly expressed on activated T cells and platelets [see 61]. CD40L is cryptic in unstimulated platelets, but rapidly becomes exposed on the platelet surface after stimulation where it is subsequently cleaved, producing a soluble hydrolytic product termed sCD40L [61]. We have shown that mice lacking CD40L have a thrombosis phenotype and that normal thrombosis is regained upon infusion of sCD40L [62]. Interestingly, sCD40L, in addition to being a ligand for CD40, is also a ligand for GP IIb-IIIa, a reaction that depends upon its KGD sequence, a known GP IIb-IIIa binding motif. sCD40L also triggers outside-in signaling by tyrosine phosphorylation of GP IIIa, a reaction which is defective in the platelets from the DiYF mouse [63]. While inhibition of primary platelet aggregation and this secondary aggregation mechanism are both inhibited by GP IIb-IIIa antagonists, a potential drug target is the metalloproteinase responsible for CD40L cleavage. Another protein released upon platelet activation that functions to consolidate platelet thrombi is Gas6. Gene targeting of Gas6 also demonstrates a thrombosis phenotype [64]. Gas6 binds to three receptors on platelets, Tyro3, Axl and Mer, but genetic targeting of any one unexpectedly inhibits Gas6-induced platelet stimulation. However, as Gas6 also induces tyrosine phosphorylation of GP IIIa, apparently by a mechanism independent of binding to the integrin, it has been proposed that Gas-6 signaling could be therapeutically regulated through inhibition of Gas6-GP IIb-IIIa cross-talk [65].

Platelet–platelet contacts induce the activation of additional signaling mechanisms which are involved in aggregate stability. One involves Eph kinases and ephrins, specifically EphA4 and ephrinB1, which through receptor ligand interactions on the platelet surface enhance the binding of GP IIb-IIIa to immobilized fibrinogen in the presence of physiological agonists [66]. Recent work from our laboratory using both

oligonucleotide-based microarray analyses and mass spectrometric proteomics techniques has identified two additional receptor families that are involved. One involves two members of the SLAM family of adhesion receptors, SLAM and CD84; the other involves a novel protein termed PEAR1 (N. Nanda, M. Hart and D.R. Phillips, unpublished data). All proteins are exposed on the surfaces of unstimulated platelets and signal secondary to GP IIb-IIIa-mediated platelet–platelet contacts by becoming tyrosine phosphorylated. Therapeutic targeting of one or more of these secondary aggregation receptor systems in platelets is an attractive possibility as they appear to have a greater effect on thrombosis than they do on hemostasis.

### *Adhesion receptors*

Several platelet adhesion receptors have been identified but we will focus on GPVI and GPIbα, the adhesion receptors that are not only involved in the adhesion of platelets to the highly thrombogenic fibrillar collagens in the vessel wall exposed following vascular injury but also contribute to platelet activation. Both receptors are attractive drug discovery targets as both are platelet specific. Under the high shear rates encountered in coronary and carotid arteries, the binding of VWF to the collagen surface triggers a transient interaction with GPIbα that allows for a more stable interaction of the platelet with the collagen surface via at least two collagen receptors, integrin $\alpha_2\beta_1$ and GPVI. Recent findings indicate that GPIbα and $\alpha_2\beta_1$ preferentially contribute to the adhesion process whereas engagement of GPVI triggers signaling events leading to platelet activation [67]. GPVI is non-covalently associated with the Fc receptor γ-chain and signals through the platelet via stimulation of multiple non-receptor tyrosine kinases. Interestingly, part of this signaling pathway may be common to GPIbα activation, and signals coming from these two receptors contribute to the activation of the receptor function of GP IIb-IIIa and platelet aggregation. Modulation of the GPVI receptor function is becoming an attractive target as platelets from GPVI-deficient animals, human platelets expressing low levels of GPVI, or platelets treated with a GPVI antibody, while unable to support thrombus growth [67–69], are nonetheless able to adhere on the collagen surface (via $\alpha_2\beta_1$ integrin and potentially another collagen receptor [70]) minimizing the effects on hemostasis [71,72]. GPIbα is a high shear rate-dependent thrombosis receptor that affects recruitment of platelets at sites of vascular injury (on the collagen present in the subendothelium and on adhering platelets) with minor impact on venous thrombotic process. Modulation of the VWF/GPIbα axis has been the subject of many investigations with promising animal experimental results, but severe thrombocytopenia has been associated with the use of antibodies against GPIbα, thus reducing the general interest of the scientific community for several years. Nevertheless, novel strategies targeting the VWF/GPIbα axis through snake venom proteins cleaving GPIbα, VWF peptides or antibodies against VWF are reviving this strategy.

### *Signaling pathways*

The extensive repertoire of platelet functions, while initiated by receptors, is regulated by signal transduction pathways. While these pathways have not been known as drug discovery targets, two observations suggest that they are worthy of consideration. First, the remarkable success of Gleevec, an Abelson tyrosine kinase inhibitor, has proven efficacy in the treatment of chronic myelogeneous leukemia and other cancers. While the multiple functions of this kinase in diverse cell types predicted toxicity, clinical data have shown that the benefits far outweigh the liabilities. The success of this drug suggests that signal transduction pathways, though redundant for multiple signaling systems in diverse cell types, are worthy of consideration as therapeutic drug discovery targets. This conclusion is supported by the analysis of numerous gene-targeted mouse strains which have led to the surprising conclusion that the phenotype achieved by the disruption of any specific gene is often limited, even for genes involved in signal transduction. A second observation is that the platelet stimuli often induce diverse responses. For example, any one of the primary platelet agonists are capable of producing a spectrum of responses which could have pathological implications, e.g. aggregation caused by the activation of the receptor function of GP IIb-IIIa, expression of procoagulant activity of prothrombinase or FXase to catalyze the production of thrombin, generation of vasoactive substances such as TXA2 and serotonin to induce vasoconstriction, release of proinflammatory proteins like sCD40L, RANTES and TGF-β to affect vascular inflammation including the progression of atherosclerosis, the release of growth factors such as PDGF to affect vascular remodeling, and the activation of secondary aggregation receptors such as SLAM, CD84 and the ephrins to stabilize thombi and cause vascular occlusions. As many of these responses would be expected to be regulated by a specific pathway, it is reasonable to expect that these responses could be individually regulated. If true, this approach could inhibit platelet-dependent pathologies without compromising primary hemostasis. One potential example is PI-3 kinase and the regulation of the adhesive function of GP IIb-IIIa [73].

The roles of secondary signaling events downstream of platelet surface receptors have been elucidated through gene-targeting studies in mice, and subsequent evaluation of their platelet phenotypes using both *in vitro* and *in vivo* techniques. As certain key platelet agonists such as ADP, thrombin, and TXA2 all activate platelets through G protein-coupled receptors, genetic targeting of individual α-subunits of G proteins has been a successful strategy in studying platelet signaling downstream of receptor activation. Characterization of platelets from mice lacking $G_q$, $G_{12}$ and $G_{13}$ identified these three proteins as key mediators for ADP and $TXA_2$ receptors [27,74–76]. Targeting of additional subunits ($G_z$, $G_{15}$, and $G_{12}$) showed little or no effect on platelet phenotype, which could be due to lack of coupling of these subunits to critical platelet receptors, or due to redundancy in the signaling pathways. Thrombin signaling has been shown to be affected by lack of $G_i$, $G_q$, and

$G_{13}$, to varying extents. In mice lacking both $G_q$ and $G_{12}$, no platelet activation was possible by ADP, $TXA_2$, or thrombin [27], suggesting that at least $G_q$ or $G_{13}$ is required to induce some activation, and that activation of $G_i$-type proteins alone is not sufficient for activation of mouse platelets. In these $G_q/G_{12}$ double-deficient platelets, adhesion of platelets to collagen was not affected; however, aggregation in response to collagen fibrils as well as formation of stable aggregates on collagen-coated surfaces was completely eliminated. In addition to targeting of G protein subunits, targeting molecules involved in kinase signaling pathways have resulted in mice with impaired platelet functions, which is not unexpected given the fact that kinases of the src family (Csk, src) have been shown to be physically associated with the cytoplasmic domain of GP IIb-IIIa [77]. The platelet phenotype of mice lacking the tyrosine kinase syk, critical for downstream signaling through the collagen receptor GPVI, and also activated during outside-in signaling and activation of GP IIb-IIIa, exhibited defects in platelet activation induced by ADP ± epinephrine [78]. Mice lacking the adapter protein SLP-76, which is on the syk signaling pathway, have been shown to have defects in GP IIb-IIIa signaling and collagen receptor responses [79]. The important role of downstream signaling molecules identified through gene-targeting studies may provide new opportunities for therapeutic intervention for blockade of platelet activation, thrombus formation, and adhesion.

## Evolving paradigm on the relationship of thrombosis to inflammation

Not only are platelets critical players in mediating thrombosis, but recently their role in inflammation has become more appreciated. Although it is widely accepted that inflammatory activities that orchestrate the progression of atherosclerosis are derived from 'traditional' inflammatory cells such as monocytes and neutrophils, emerging data suggest that products released from platelets during thrombosis are actively involved in this process and that platelets are a primary source of inflammatory proteins within the circulation. For example, Sohober et al. [80] reported that platelets deposit RANTES onto endothelial cells in the injured vessel wall, and that this interaction is mediated by P-selectin, a surface receptor mediating the attachment of platelets to leukocytes and endothelium. Additional work from this laboratory showed that infusion of activated platelets into the $apoE^{-/-}$ mouse greatly enhanced the rate of atherosclerotic lesion progression [81]. The exposure of P-selectin following platelet activation is a key mediator of platelet–leukocyte interaction, and facilitates atherosclerotic lesion development, as demonstrated by Burger and Wagner [82]. In patients with acute MI, platelet–leukocyte interaction is increased compared with controls, and P-selectin levels have been shown to remain increased for at least a month following initial presentation in ACS patients with non-ST segment elevation [83], and even in patients with stable coronary artery disease. Antiplatelet therapy with a $P2Y_{12}$ antagonist plus aspirin was shown to decrease platelet–monocyte interactions that occur after coronary stenting [84], an effect not observed with anticoagulants plus aspirin, suggesting that $P2Y_{12}$ antagonism had an anti-inflammatory effect distinct from its antithrombotic mode of action. Targeting of CD40L, another platelet-derived inflammatory protein, either through a blocking antibody [85] or via gene-targeting [86], greatly inhibited lesion progression in either $LDLR^{-/-}$ or $apoE^{-/-}$ mice, respectively. Soluble CD40L (sCD40L), the shed hydrolytic product of CD40L, 95% of which is platelet-derived, has been shown to be a primary risk factor for atherosclerosis/ thrombosis [87]. In addition, the binding of platelet-derived sCD40L to endothelial cells can lead to the expression of tissue factor, a potent procoagulant. Clopidogrel has been shown to inhibit ADP-induced CD40L expression, and to lower CD40L levels in patients undergoing PCI [88], while aspirin does not. Thus, some forms of antiplatelet therapy, including $P2Y_{12}$ inhibition, can inhibit platelet pro-inflammatory responses. Finally, recent data show that targeting of GP Ib-IX-V complex, a platelet adhesion receptor, in the $apoE^{-/-}$ mouse blocks leukocyte recruitment and the development of atherosclerotic lesions [89]. Thus, studies of the role of platelets in inflammation may provide new potential targets for CVD through inhibition of atherosclerosis and/or thrombosis.

## Platelet monitoring

The pharmaceutical industry and drug approval agencies expect that the early inroads into personalized medicine in the administration of selected chemotherapies will ultimately extend to all drug classes. These early examples include screening for HER2 positive individuals in the treatment of breast cancer with Herceptin and the screening for EGF receptor for the treatment of lung cancer with gefitinib [90]. More generally, by the time this manuscript is published, the CYP450 screen will most likely be available to detect the various isoforms of P450 which will be useful in projecting drug levels in individuals treated with a wide variety of drugs. While such strategies will be of value in selecting and dosing antithrombotics, particularly for chronic use, access to blood of patients treated with antithrombotics continues to provide the best opportunity for monitoring the effect of any therapy or any combination of therapies on each patient being treated. While suitable assays are available to monitor anticoagulants, the monitoring of antiplatelet drugs is not routinely performed.

Evaluations of data from the current antithrombotics and their limitations have defined the assays required to bring personalized medicine to the patient treated with antiplatelet drugs to prevent arterial thrombosis. First, platelet function should be monitored in the context of thrombosis. Platelet thrombosis *in vivo* is initiated by adhesive proteins exposed on the vessel wall and stable thrombi result following adhesion, activation, aggregation, and thrombus stabilization, all occurring under conditions of shear. As thrombus stability is one of the issues, continuous monitoring of thrombus formation is essential for determining the effects of drugs that effect targets

involved in thrombus stability, for example, in prostanoid metabolism and in ADP release. Although methods currently available such as light transmittance aggregometry, the Ultegra Rapid Platelet Function Assay and platelet activation markers such as P-selectin expression are effective in monitoring the ability of end products of any one of these pathways to activate platelets, they are ineffective in monitoring thrombosis, the physiological response of platelets to thrombogenic surfaces under shear. Furthermore, while the PFA-100 device is capable of monitoring the time required for a platelet plug to form in apertures coated with ADP or collagen, it does not provide a continuous monitoring of the thrombotic process. A second requirement for the personalized monitoring of drugs to prevent arterial thrombosis is that it should not only be responsive to diverse drug classes but also be capable of determining the net effect on thrombosis achieved by combinations of antithrombotic therapies. At the present time, the four drug classes used to treat patients at risk for arterial thrombosis, aspirin, GP IIb-IIIa antagonists, clopidogrel and anticoagulants, are used in combinations not properly evaluated for their net effect on thrombosis. Drugs against additional platelet and coagulation protein targets will become available. Required are technologies that will readily permit evaluation of how combinations of these drugs affect thrombosis in patients receiving these drugs. A third requirement is that the method must be capable of monitoring individual differences in response to therapy. As outlined above, individual differences in response to aspirin and clopidogrel have been observed – differences which appear to affect clinical outcome. While currently available techniques such as light transmittance aggregometry, Ultegra, or PFA-100 are useful in monitoring responsiveness to both of these drugs as monotherapy, they are ill-suited to measure individual differences when combinations of drugs are employed, the emerging norm. It is also anticipated that this problem would be amplified when drugs against additional targets would be introduced. Finally, as the anticoagulants routinely used in blood collection such as citrate or direct thrombin inhibitors such as PPACK affect thrombosis, the monitoring method must be capable of assaying non-anticoagulated samples of blood. Perhaps the best example of anticoagulant interference in antithrombotic monitoring is in the development of GP IIb-IIIa antagonists where citrate anticoagulation markedly overestimates antiaggregatory activity [91]. The optimal method for monitoring individual thrombotic potential must be capable of either performing the assay in the absence of anticoagulants or of being able to determine how any given anticoagulant affects the assay.

Use of perfusion chamber technology has perhaps provided the best hope of measurement of the thrombotic potential of individuals being treated with combinations of antithrombotic drugs. Perfusion chambers were designed 30 years ago in order to characterize the thrombotic process under shear conditions. The different types of perfusion chambers described in the literature can be classified according to their geometry (circular, annular, flat chambers) or the surfaces (blood vessels, isolated proteins) exposed to flowing blood. These techniques confer the advantage of studying platelet interactions with a thrombogenic surface under specific conditions of shear rate with either non-anticoagulated or anticoagulated blood. Major contributions to the field of thrombosis have originated from use of perfusion chambers. For example, the critical role of VWF and its interactions with GP Ib and GP IIb-IIIa to mediate platelet adhesion and thrombus growth under arterial shear rates, the involvement of GP VI and of the integrin $\alpha 2\beta 1$ in mediating platelet adhesion and activation on collagen. Inhibitors of $P2Y_{12}$ and Cox-1 have also demonstrated antithrombotic activities in this system [92]. However, perfusion chambers are mostly utilized by academic institutions or by pharmaceutical and biotechnology companies in order to identify or validate targets and to develop antithrombotic drugs. Several limiting factors have prevented their use as a bedside device for monitoring drug efficacy in clinical trials – the skill required to determine thrombus size was not readily available in clinical settings; quick readouts for the patient were not available; end point quantifications left investigators without knowledge of the kinetics of thrombus formation, the more critical information.

Results from several laboratories, however, have made progress in modifying these devices to circumvent these difficulties. Figures 3 and 4 illustrate the utility of monitoring the kinetics of thrombosis in perfusion chamber assays. In one instance, using non-anticoagulated samples of blood, we have shown that inhibition of $P2Y_{12}$, Cox-1, or FXa did not significantly reduce thrombus size after a 4-min perfusion period over a collagen-coated surface. However, when more than one of these targets was inhibited, pronounced antithrombotic activity was observed. In another experiment, when human blood was anticoagulated with an FXa inhibitor and perfused through a chamber in the real-time assay, we observed that $P2Y_{12}$ antagonism with clopidogrel did not affect the

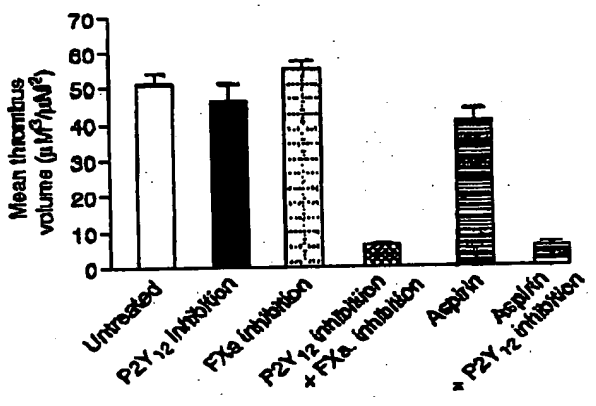


**Fig. 3.** Synergism between $P2Y_{12}$ antagonism, Factor Xa inhibition and aspirin. As indicated unanticoagulated blood was treated with an inhibitor for $P2Y_{12}$ (100 μM 2MeSAMP) or Factor Xa (10 μM C921–78). Aspirin-treated was from aspirin-treated individuals. The treated blood was perfused through a chamber coated with type III collagen at 1000 $s^{-1}$ for 4 min and quantified as described [23].

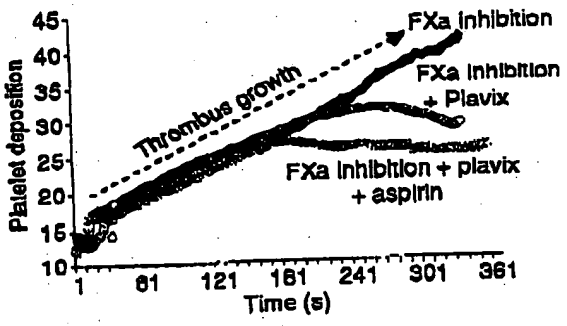


**Fig. 4.** Synergism between aspirin and $P2Y_{12}$ inhibition in blocking thrombus growth. Blood from a control individual, an individual treated with clopidogrel, or an individual treated with aspirin and clopidogrel was anticoagulated with a Factor Xa inhibitor, treated with rhodamine 6G to label platelets, and then perfused through a chamber coated with type III collagen at 1000 $s^{-1}$. The continuous accumulation of fluorescence was used to quantify platelet thrombus formation.

initial thrombus growth triggered by fibrillar type III collagen under arterial shear rates. However, clopidogrel caused the thrombi formed during the first 3 min of perfusion to dissociate. Control thrombi formed in the absence of clopidogrel were stable and continued to grow. This demonstrates the limitations of end point analysis as the measured antithrombotic activity is dependent on the time of analysis. Retrospectively, this explains an apparent discrepancy found in the evaluation of the phenotype of $P2Y_{12}^{-/-}$ mice [57]. $P2Y_{12}^{-/-}$ mice demonstrate a cyclic thrombotic process *in vivo*, but only a qualitative difference (i.e. more loosely packed thrombi) was observed after perfusion of non-anticoagulated blood for 2.5 min over type III collagen. Evaluation of mono- and combination therapies in this assay confirmed the antithrombotic efficacy of the different anti-platelet therapies, with GP IIb-IIIa antagonists being inhibitors of thrombus growth, aspirin and $P2Y_{12}$ antagonist destabilization agents, the combination aspirin + $P2Y_{12}$ antagonism showing a faster destabilization activity (Fig. 4). Thus, perfusion chamber technology is suited to meet the requirements of personalized medicine for individuals receiving antithrombotic therapies as the measurement is on thrombosis, it is responsive to diverse drug classes and it can be performed in the absence of anticoagulants. Future discoveries are required to adapt such technologies to devices that are readily available to individual patients. Finally, several laboratories have reported measurements of the inflammatory activities of platelets, e.g. sCD40L plasma levels, P-selectin expression, formation of platelet-leukocyte complexes. As recent data show that the inflammatory activity of platelets is important in the progression of atherosclerosis, it would also be desirable to develop methods to rapidly quantitate the platelet inflammatory activity in patients.

Application of this personalized medicine approach to antithrombotic therapies does have significant hurdles to overcome before it can be used to reliably modify therapy. First, a bedside monitor of the thrombotic potential of individual patients needs to be developed. Second, recognizing that individuals will undoubtedly be heterogeneous with respect to vessel wall thrombogenicity, including the local shear environment, results using this device need to be correlated with clinical outcomes.

## Conclusions

The current repertoire of drugs for the treatment of patients at risk for arterial thrombosis (e.g. ACS, diabetes, poststroke, peripheral artery disease, post-AMI) currently includes four classes of drugs - aspirin, GP IIb-IIIa antagonists, thienopyridines, and anticoagulants. Although each of these drug classes has proven efficacies for different indications, each has limitations that continue to permit thrombotic events during their use. In addition, emerging data suggest that a significant percentage of individuals treated with aspirin or clopidogrel do not receive the expected therapeutic benefit from therapy because of a decreased responsiveness by their platelets. Future directions in addressing these limitations will proceed in two parallel directions. On the one hand, it can be anticipated that new drugs, either offering improvements against known, validated targets, or against recently identified targets, will be forthcoming. Recognizing that platelets are now known to be directly involved in vascular inflammation including that which leads to the progression of atherosclerotic disease, it can be anticipated that some of these new therapeutic strategies will not only better address arterial thrombosis, but also inhibit the ability of platelets to deliver inflammatory proteins and growth factors which affect atherosclerotic lesion development. On the other hand, it has now become apparent that improvements are required in the devices used to monitor the thrombotic potential of individuals receiving therapy, both for the development of new antithrombotic drugs and to measure the effectiveness of combined antithrombotic therapies. It would appear that the most effective device is that which measures thrombosis in real time, is accessible to the patient at the point of drug administration, and can be performed in the absence of anticoagulation. Such a device would be capable of monitoring the activities of new classes of antithrombotics, of measuring variances of individual responses, and in evaluating the effectiveness of combined antithrombotic therapies.

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